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ORIGINAL ARTICLE

Benefit of Kupffer cell modulation with glycine versus Kupffer cell depletion after liver transplantation in the rat: effects on postischemic reperfusion injury, apoptotic cell death graft regeneration and survival*

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Summary

Inhibition or destruction of Kupffer cells (KC) may protect against ischemia-reperfusion (IR) induced primary graft nonfunction (PNF) in liver transplantation. Besides KC activation, PNF is characterized by microvascular perfusion failure, intrahepatic leukocyte accumulation, cell death and hepatocellular dysfunction. KCs can be inactivated by different agents including gadolinium chloride ($GdCl_3$), methyl palmitate (MP) and glycine. The effects of three KC inactivators on IR-injury after rat liver transplantation were compared in the present study. Lewis liver donors were treated with $GdCl_3$, MP, glycine or saline (control). Liver grafts were transplanted following 24 h storage (UW solution). KC populations and IR damage were assessed by histologic analysis, quantitative real-time polymerase chain reaction (RT-PCR) and intravital microscopy. The number of hepatic ED-1 positive macrophages was diminished after $GdCl_3$ ($114.8 \pm 4.4/mm^2$ liver tissue) and MP treatment (176.0 ± 5.0), versus the glycine (263.9 ± 5.5) and control (272.1 ± 5.6) groups. All three treatment modalities downregulated phagocytic activity for latex particles, paralleled by reduced microvascular injury (acinar perfusion index, $GdCl_3$: 0.75 ± 0.03; MP: $0.83 \pm .03$; glycine: 0.84 ± 0.03; 0.63 ± 0.03). Quantitative RT-PCR revealed elevated myeloperoxidase mRNA after glycine versus $GdCl_3$ and MP pretreatment (3.2- and 3.4-fold, $P = 0.011$, respectively), without difference to controls (2.9-fold of glycine). TNFα-mRNA was reduced after glycine- (5.2-fold), $GdCl_3$- (19.7-fold), MP-treatment (39.5-fold) compared with controls. However, profound prevention of intrahepatic cell death and liver graft failure was solely achieved with glycine preconditioning. Different than $GdCl_3$ and MP, glycine modulates rather than destroys KCs. Glycine appears to preserve cell viability and to TNFα/leukocyte dependent organ regeneration capacity, which is related to increase graft survival following liver transplantation.

Introduction

Liver graft injury because of cold ischemia and reperfusion represents one of the major obstacles in liver transplantation protocols [1]. Ischemia-reperfusion (IR) injury is known to severely compromise early graft function, potentially contributing to graft loss and requirement of re-transplantation. Furthermore, it predicts long-term success after transplantation related to early induction of immune processes [2,3], becoming apparent as ischemic

type biliary lesions or graft rejection. Despite intense research on the development of new therapeutic strategies to counteract IR-dependent mechanisms [4], only a small fraction of the concepts have been introduced into clinical practice.

The IR-injury in liver grafts is characterized by a sequence of morphologic changes. These include disturbances of microvascular perfusion secondary to injury of the sinusoidal lining signified by endothelial cells and Kupffer cell (KC) disturbances [5,6], intrahepatic accumulation of white blood cells (WBCs) [7], and impaired hepatocellular function [8,9]. In addition, apoptotic cell death has been acknowledged as an important mechanism related to cellular IR injury [5,10]. Nonetheless, activation of KCs has been identified as a key event in the initiation and perpetuation of the IR injury. Indeed, activated KCs represent a major intrahepatic source of potent mediators such as reactive oxygen species, tumor necrosis factor (TNF)α, cytokines [interleukin (IL)-6], eicosanoids and chemokines. However, the detrimental or protective function of some mediators remains to be identified. For these reasons substances have been characterized to control KCs, either by destruction or by modulation. Among these substances are gadolinium chloride ($GdCl_3$), methyl palmitate (MP) and glycine. $GdCl_3$ is a rare earth metal salt with high similarity in crystal radii to calcium. $GdCl_3$ can replace calcium ions, potentially interfering with calcium uptake and calcium-dependent cellular processes [11] including phagocytosis and proteolysis activation. $GdCl_3$ causes KC elimination, without described effects on other hepatic cell populations [12]. MP, a non-hydrolyzable fatty acid ester, exerts its effects by inhibiting KC phagocytic activity, and by reducing the immunologic response to foreign antigens [13–15]. Although the underlying mechanisms remain somewhat speculative, MP integration into cell membranes seems to change membrane characteristics to provide a protective effect. The third substance of importance in the present study is glycine, which inhibits calcium influx into cells by binding to a glycine-gated chloride channel [16,17]. Activation of this channel by glycine leads to hyperpolarization of the cell membrane by permitting chloride influx into the cell. This blunts calcium-influx into the cell by limiting the opening of voltage-gated channels [16], which decreases KC activity and protects liver grafts [18,19]. However, although there is clear evidence for the detrimental effect of KCs after warm ischemia *in vivo*, increasing evidence in recent publications strongly challenge this pathologic concept in the context of cold ischemia and reperfusion in liver transplantation [20,21].

Although KC inhibition by $GdCl_3$, MP and glycine have been evaluated in several experimental models in the past, direct comparisons after liver transplantation are rare, and existing studies were performed in highly specialized *in vitro* models or with short preservation times [19,22]. The purpose of the present study was to evaluate these three KC inhibitors on early graft IR injury, intrahepatic cellular apoptosis, regeneration stimuli and survival after liver transplantation in the rat.

Material and methods

Experimental groups

Four experimental groups were compared. Each group was divided into two sets of studies, consisting of one set of intravital microscopy experiments, and a second set of survival experiments. Separation of these study sets permitted the exclusion of influences of the intravital microscopy on animal survival following transplantation. In one treatment group donor animals were pretreated with a single intravenous injection of $GdCl_3$ (10 mg/kg, 1 ml; Sigma Chemicals, Deisenhofen, Germany) 24 h before graft harvesting. In a second treatment group organ donors were treated in the same way with a bolus intravenous injection of MP (300 mg/kg; Sigma Chemicals). MP was prepared as a stock emulsion by sonification of 100 mg MP/ml Ringers solution containing 0.2% Tween-20 and 5% dextrose. In the third treatment group donors were pretreated with glycine (300 mM, 1 ml; Merck, Darmstadt, Germany) as a 1 h i.v. infusion preceding the graft UW perfusion. All treatment regimen were reproduced with minor modifications from previous publications [15,22]. The fourth group, pretreated with an equal volume of physiologic saline 24 h prior to reperfusion, served as the control group.

Additional experiments with glycine treatment 24 h before, and $GdCl_3$ and MP treatment 1 h before graft harvest were performed to exclude specific effects of timing of donor pretreatment. The determination of survival for 7 days showed no differences between the groups. Results were excluded from further analyses.

Surgical procedure

Syngeneic orthotopic liver transplantation, including sequential arterial reconstruction, was performed in male Lewis rats weighing 170–190 g (Charles River Wiga, Sulzfeld, Germany). Details of the surgical technique have been described in detail previously [23,24]. The cold ischemia time was 24 h in UW solution (4 °C; Du Pont de Nemours, Bad Homburg, Germany). Bile flow was monitored after insertion of a polyethylene tube (PE-50, 0.58 mm inside diameter; Portex, Hythe, UK) into the common bile duct. Liver tissue specimens were collected after a 90 min interval of intravital microscopy (see below) or at 360 and 540 min after graft reperfusion. In

experiments, determining animal survival, the bile duct was reconstructed. Animals were given free access to food and water after the surgical procedure. According to previous studies in syngeneic rat liver transplantation experiments [23], and our own observations, surgery related technical complications and early graft failure as causes of death can be excluded within 7 days postreperfusion. Therefore, all recipients surviving 7 days were assumed to be long-term survivors. All experiments were carried out in accordance with the German legislation on animal protection (permission no 621-2531.1-13/99, Government of Oberpfalz, Germany) and with the 'Principles of Laboratory Animal Care' (NIH publication no. 86–23, revised 1985).

Intravital fluorescence microscopy and video analysis

After complete graft revascularization and recovery of the animals from the anhepatic period, *in vivo* epi-illumination microscopy was performed from 30 to 90 min after portal graft reperfusion, as described previously in detail [24]. Hepatic acinar and sinusoidal perfusion was assessed after injection of sodium fluorescein (1 μmol/kg; Merck). Eighty minutes after reperfusion, phagocytic activity was quantified after an intra-arterial bolus injection of fluorescent latex particles (3×10^8 yellow-green 1.1 μm diameter particles/kg; Polysciences Inc., Warrington, PA, USA). Video-registered microscopy sequences were assessed off-line by frame-to-frame analysis. Estimation of acinar perfusion was performed in lower magnification (240×; 50–100 acini) classifying acini as completely perfused, irregularly perfused, or nonperfused, depending on the distribution of sodium fluorescein. In accordance with previous studies [24], the acinar perfusion index was calculated as (well-perfused acini + $0.5 \times$ irregularly-perfused acini)/total number of acini.

In addition, sinusoidal perfusion and KC phagocytic activity were assessed (600× magnification) in randomly selected individual acini at 35 until 80 min after graft reperfusion. Sinusoidal perfusion was expressed as the percentage of nonperfused of all observed sinusoids in each subacinar zone (periportal, midzonal, pericentral). Of note, distribution characteristics of fluorescent compounds permit the quantification of sinusoidal perfusion exclusively within perfused acini.

Kupffer cell phagocytic activity was analyzed continuously from 80 to 90 min after reperfusion in 15–20 randomly selected microscopic fields (405×540 μm). The percentage of moving latex-particles per field (8–12 s of observation) was determined. The measurements started in each experiment with appearance of the first particle within the sinusoids. Latex particles in postsinusoidal venules were not quantified.

Graft excretory function

For evaluation of graft excretory function, the total volume of bile secreted during the initial 90-min reperfusion interval was measured. Bile production was standardized to milliliter per 100 g liver tissue, per 90 min. Bile acid content (μmol/100 g/90 min) was assessed using the 3-α-dehydrogenase test [25].

Immunohistochemistry

To assess KC numbers, paraffin-embedded liver tissue sections were stained for ED-1 (CD68) with a CD68-specific antibody (BMA, Basel, Switzerland). Visualization was achieved with the DAKO streptavidin–biotin complex staining method (DAKO, Hamburg, Germany), and the chromogen 3,3′-diaminobenzidin-tetrahydrochloride. Sections were counterstained with hematoxylin (Merck). Cells were counted in 10 randomly chosen high-power fields in each tissue section, with one sections of the large left and right liver lobe per animal, and expressed as positive cells per square millimeter liver tissue.

Terminal UDP nick end labeling assay

The number of apoptotic cells was determined by standard terminal UDP nick end labeling (TUNEL) staining (Boehringer, Mannheim, Germany) of liver tissue sections, according to the manufacturers instructions. TUNEL was visualized using 3,3′-diaminobenzidin-tetrahydrochloride. TUNEL positive cells were counted in 10 randomly chosen high-power fields in each tissue section, with one sections of each, the large left and right, liver lobe per animal, and expressed as positive cells per square millimeter liver tissue.

Western blotting

Detection of caspase 3 (CPP32) and its active cleavage product (p20 subunit) in hepatic tissue was performed by Western blot analysis. Tissue was minced in buffer containing 1% nonylphenoxypolyethoxyethanol, 0.5% sodium deoxycholate and 0.1% SDS in PBS supplemented with a protease-inhibitor mixture containing aprotinin (100 U/ml), sodium orthovanadate (184 mg/ml) and phenylmethylsulfonyl fluoride (100 mg/ml). The protein concentrations were determined using a 'BC assay: protein quantitation kit' (Uptima, Interchim, Montlucon, France). Similar protein quantities in electrophoresis gels were controlled by actin determinations. SDS gel-electrophoresis and protein transfer onto nitrocellulose membranes was followed by incubation with caspase 3 (p20) and actin specific antibodies (24 h/4 °C). For

visualization horseradish peroxidase (HRP) conjungated secondary antibodies were used (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was used to measure intrahepatic messenger RNA (mRNA) expression of CD163 (specific for mononuclear cell), myeloperoxidase (specific for granulocytes), and of TNF-α. Tissue samples were homogenized in TRIzol reagent (Invitrogen, Karlsruhe, Germany) and total RNA was extracted. To exclude potential genomic DNA contamination the samples were treated with DNAseI (DNAfree, Ambion, Huntingdon, UK). Reverse transcription was performed with the first strand cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany) using oligo-dT primers. PCR primers were designed from the corresponding cDNA sequences from the GeneBank (numbers: CD163: XM_232342; MPO: XM_220830; TNFa: NM_012675). RT-PCR was performed using a Light Cycler and the Fast Start cDNA SYBR Green Kit (Roche Diagnostics) with 0.5 μM of primers and 2 μl cDNA template. Detection temperature and $MgCl_2$ concentration were optimized for each primer. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The specificity of the PCR was confirmed by sequencing the products of each primer pair, the absence of nonspecific amplification was confirmed by agarose gel electrophoresis. PCR levels are presented as relations of arbitary units.

Statistical analysis

Data are presented as mean ± SEM. Non-normally distributed data was calculated after rank transformation. Depending on the number of measurements per experiment one-way ANOVA or univariate ANOVA was used for calculations, and Tukey-HSD test was used for *post hoc* comparisons between the groups. $P < 0.05$ was considered significant. Survival was estimated using Kaplan–Meier/log-rank analysis. As previously described [26], kinetic of latex particle adherence was calculated using a general-linear model with log-transformed percentages of moving latex particles as the dependent variable. All calculations were performed by use of the SPSS procedures UNIVARIATE and GLM (SPSS® Inc, Chicago, IL, USA).

Results

Effect of KC inhibitor pretreatment on KC numbers and function

Immunohistological ED-1 staining of liver grafts revealed clear differences in the number of positive cells between the groups (Fig. 1). $GdCl_3$ pretreatment of the grafts resulted in a marked decrease in the total number of ED-1 positive cells versus controls ($P < 0.001$). MP treatment caused a less pronounced decrease in ED-1 positive cells ($P < 0.001$ versus controls and versus $GdCl_3$,), however, glycine had no significant effect compared with controls. These results was furthermore supported by measurements of the monocyte-specific epitope CD163 with quantitative RT-PCR, which showed a highly significant decrease (440-fold, $P < 0.001$) after $GdCl_3$ and MP-pretreatment of liver grafts in contrast to controls, which was not evident after glycine treatment (7-fold decrease, $P = 0.07$). Together, these data indicated a decrease in the number of KCs is after $GdCl_3$ or MP treatment, and minor effects of glycine on the KC population.

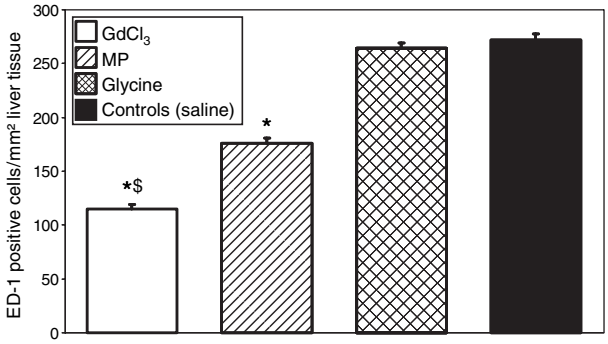

Figure 1 Immunohistological staining of ED-1 at 90 min after reperfusion as assessed with light microscopy. Pretreatment of organ donors with $GdCl_3$ or MP resulted in a significant decrease in intrahepatic ED-1 positive cells, compared with glycine pretreatment or controls. Data are presented as the mean ± SEM. *$P < 0.05$ vs. controls.

Live analysis of injected latex particles revealed a progressive decrease in their movement in all groups within the transplanted liver (Fig. 2). This decrease in particle movement reflects phagocytic activity in the liver, which is primarily because of KCs [24,26]. When making a closer comparison of the mean kinetic curves and 95% confidence intervals of the different groups, particle movement decreased faster in the control group versus groups pretreated with $GdCl_3$, MP and glycine. Therefore, these data suggest that KC phagocytic activity is lowered by each of the KC-inhibitors used in our experiments, most pronounced after profound destruction of KCs with $GdCl_3$.

Effects of KC-inhibitor pretreatment on macrohemodynamics and early hepatocellular bile excretion

Parameters which fundamentally affect early graft function and microhemodynamics, such of donor and recipient weight, liver graft weight and anhepatic period showed no statistical differences between the different groups and between the two sets of experiments (intravital microscopy

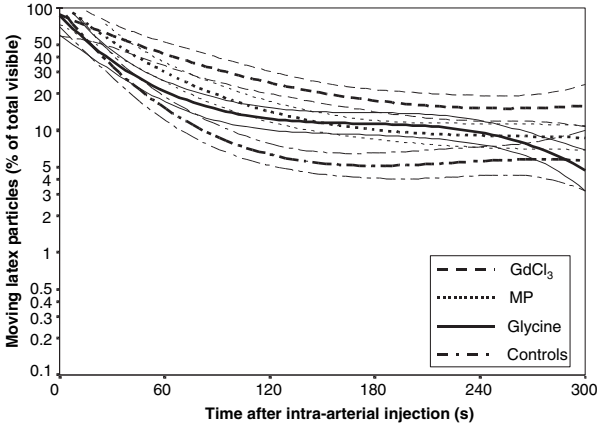

Figure 2 Phagocytosis of latex particles after a single bolus intra-arterial injection at 80 min after graft reperfusion in IVM experiments. Curves represent mean percentages of moving particles relative to all visible particles ± 95% confidence interval. Analysis reveal a faster decline in particle movement in controls, compared with glycine, MP or $GdCl_3$ pretreatment. As the decelerated velocity in particle movement is an indicator of increased phagocytic activity, these results suggest reduced phagocytic activity in all groups pretreated with KC inactivators, most pronounced after profound depletion of KCs with $GdCl_3$.

and survival experiments) (Table 1). However, mean arterial pressure during the intravital microscopy varied between 58.4 and 64.9 mmHg in the different groups and thus reflected depressed macrohemodynamic stability after liver transplantation. This effect was similar in all groups independent of the treatment modalities ($GdCl_3$, MP, glycine and controls). Together, from these data, any influence on early graft excretory function and microvascular graft injury in the different groups can be excluded.

Bile flow and bile acid excretion, however, is a highly sensitive indicator of early graft function in human liver transplantation [8,9], which may depend on the activation state of KCs [24]. Therefore, by inhibition of KCs changes in hepatocellular function could be expected. However, no significant difference was detectable in comparison to recipients after treatment with $GdCl_3$ (2.2 ± 0.5 ml/100 g liver tissue/90 min), MP (3.2 ± 0.6), glycine (2.8 ± 0.5), versus controls (2.8 ± 0.7). Bile acid excretion showed a tendency to higher levels after glycine pretreatment (122 ± 27.4 μmol/100 g/90 min), as compared with the $GdCl_3$ (93.5 ± 25.2), MP (101.1 ± 18.7), and control groups (91.1 ± 21.6), but this difference was not statistically different.

Effects of KC-inhibitor pretreatment microvascular graft injury

As expected, a certain percentage of hepatic acini showed complete perfusion failure. Nonetheless, the relative number of nonperfused acini in this analysis ranged between 1.6 ± 1.1% (glycine) and 14.0 ± 4.3% (controls). Calculation of the acinar perfusion-index showed that perfusion was most homogenous with glycine (0.84 ± 0.03) and MP (0.83 ± 0.03) donor organ pretreatment, versus a markedly lower index with $GdCl_3$ (0.75 ± 0.03; $P = 0.019$). However, in comparison to controls (0.63 ± 0.03), all three KC modulating substances produced more homogeneous acinar perfusion ($P < 0.007$).

Furthermore, by means of intravital microscopy the percentage of nonperfused sinusoids was determined. In the present analysis, the glycine group revealed the highest number of nonperfused sinusoids (13.2 ± 1.1%), as compared with the groups after $GdCl_3$- (10.8 ± 1.1%) and MP pretreatment (10.9 ± 1.1%) and controls (12.9 ± 0.9%), however this difference was not significant. The surprisingly high percentage of sinusoidal perfusion failure after glycine pretreatment was due to perfusion disturbances in the periportal subacinar region (18.6 ± 1.7%), when analyzing the number of nonperfused sinusoids separately for in each subacinar region.

Effects of KC- inhibitor pretreatment on cell death, organ regeneration and graft survival

The effects of KC-inhibitors on the survival of sinusoidal lining and parenchymal cells in liver grafts was determined by TUNEL staining. At 90 min after graft reperfusion, all three KC-inhibitors reduced the number of nonviable sinusoidal lining and parenchymal cells to the same extent, compared with controls (Fig. 3a,b).

These results led us to test for caspase 3 activity in these tissues at 90 min after graft reperfusion, in order to confirm the process of apoptosis. Activation of caspase 3 (CPP32) represents the terminal step in the caspase cascade, and a decisive executive step of apoptotic cell death. Once activated, caspase 3-dependent processes leading to apoptosis, such as inactivation of PARP (poly-ADP-ribose polymerase; a repair enzyme for DNA strand-breaks) cannot be reversed. Importantly, caspase 3 cleavage precedes morphological signs of apoptosis in affected cells.

Western blotting KC-inhibitor pretreated liver grafts showed reduced levels of the caspase 3 cleavage product p20 after glycine treatment, when compared with $GdCl_3$, MP at 90 min after graft reperfusion (Fig. 4). As a consequence, morphological signs of apoptosis should be different after $GdCl_3$, MP and glycine pretreatment when compared after complete execution of apoptosis. We therefore analyzed the number of TUNEL positive cells after 360 and 540 min following graft reperfusion, and indeed found significant higher levels of TUNEL positive sinusoidal lining cells (Fig. 3a) and hepatocytes (Fig. 3b)

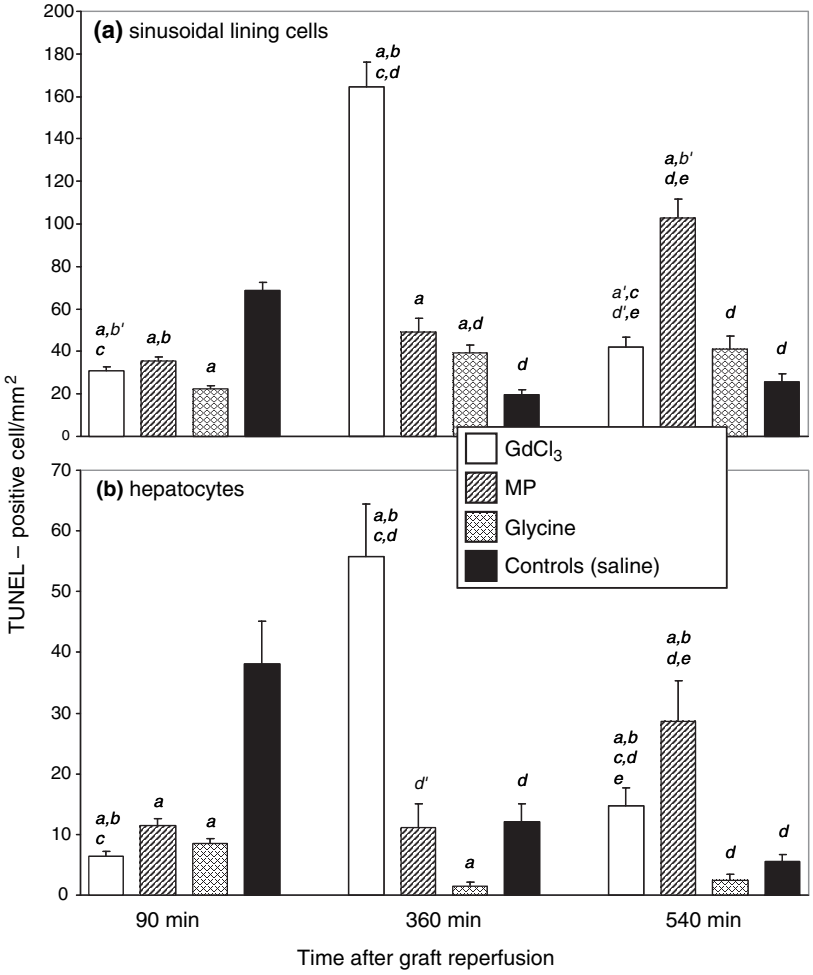

Figure 3 Quantification of apoptosis by TUNEL assay, expressed as TUNEL positive cells per mm^2. Whereas the number of TUNEL positive sinusoidal lining cells (a) and hepatocytes (b) is lowered at 360 and 540 as compared with 90 min after reperfusion in controls, positive cell counts are elevated after $GdCl_3$ and MP treatment at corresponding time points. In contrast, glycine pretreated grafts revealed stable low levels of TUNEL positive cells at 90, 360 and 540 min after reperfusion. Differences are calculated as follows: $^{a}P < 0.0001$ vs. controls; $^{a'}P < 0.05$ vs. controls; $^{b}P < 0.0001$ vs. glycine; $^{b'}P < 0.05$ vs. glycine; $^{c}P < 0.0001$ vs. MP; $^{c'}P < 0.05$ vs. MP; $^{d}P < 0.0001$ vs. 90 min level within the same group; $^{d'}P < 0.05$ vs. 90 min level within the same group; $^{e}P < 0.0001$ vs. 360 min level within the same group; $^{e'}P < 0.05$ vs. 360 min level within the same group.

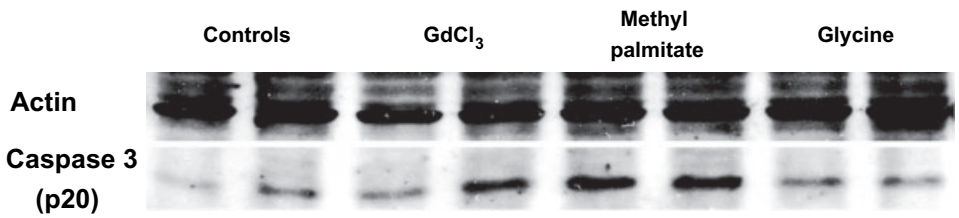

Figure 4 Caspase 3 cleavage. Representative Western blot analyses show decreased activity of cleaved caspase 3, the key enzyme for irreversible execution of apoptosis after $GdCl_3$ and MP pretreatment, compared with controls and the glycine group. The difference in activity of cleaved caspase 3 might explain the differences in the number of apoptotic cells at 360 and 540 min after portal reperfusion.

following $GdCl_3$- or MP-pretreatment numbers at 360 or 540 min after graft reperfusion as compared with 90 min levels in the same group and as compared with the control and the glycine group at corresponding time points. This effect was also observed after glycine pretreatment, but was restricted to sinusoidal lining cells and not evident for hepatocytes. This indicates a prevention of apoptosis execution by glycine in comparison to GdCl3 and MP after extended cold liver graft ischemia and reperfusion. In control animals, however, the number of apoptotic cells at time points later than 90 min was found to be lower as compared with the 90 min-value. This may indicate, that the process of apoptosis execution in the control group peaked very early after reperfusion, and apoptotic cells are not found in the liver after 6 h postreperfusion.

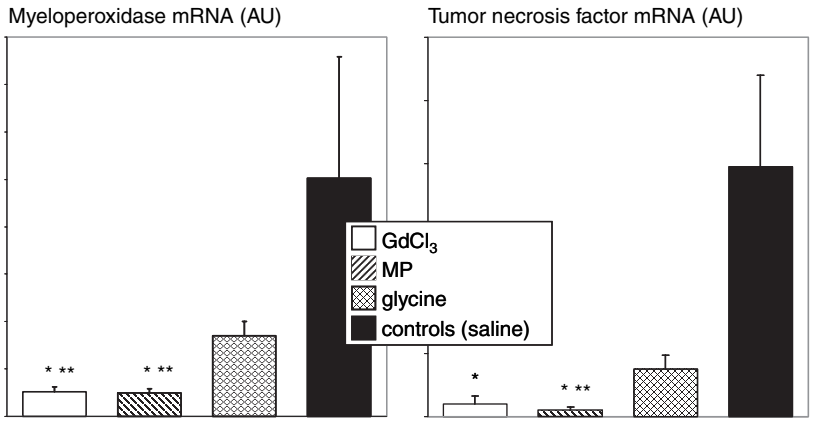

Figure 5 Intrahepatic WBC accumulation indicated by expression of myeoloperoxidase as well as TNFα expression verified by quantitative RT-PCR. Treatment with KC depleting substances ($GdCl_3$ and MP) are associated with reduced myeloperoxidase activity compared with the glycine group and controls, indicating reduced WBC accumulation following $GdCl_3$ and MP pretreatment. In parallel, TNFα expression is reduced after KC depletion. Together these data may represent a reduced potential of regenerative response of the liver grafts. Data are presented as the mean ± SEM. $*P < 0.05$ vs. controls, $**P < 0.05$ vs. glycine.

Effects of KC-inhibitor pretreatment on TNFα expression and intrahepatic granulocyte accumulation

Intrahepatic TNFα-dependent leukocyte accumulation appears to be mandatory for the regeneration capacity of hepatic tissue after cold ischemia in (partial) liver transplantation. In order to further test the cyto- and graft-protective potential of glycine in contrast to $GdCl_3$ and MP, quantitative RT-PCR was performed in hepatic tissue for detection of intragraft TNF expression. As indicator for the presence of granulocytes, relative intrahepatic MPO was in addition quantified by RT-PCR (Fig. 5). Quantitative RT-PCR revealed elevated myeloperoxidase expression after glycine compared with $GdCl_3$ (3.2-fold, $P = 0.011$) and MP pretreatment (3.4-fold, $P = 0.011$). In control grafts MPO levels were found higher than in the glycine group (2.9-fold elevation), however with no statistical difference ($P = 0.58$). In accordance with the hypothesis that TNF-expression is associated with liver graft infiltration with leukocytes we detected strongly reduced levels of TNF reduced after glycine- (5.2-fold), $GdCl_3$- (19.7-fold), MP-treatment (39.5-fold) compared with controls. Although markedly distinct differences of glycine- versus $GdCl_3$- and MP-pretreated grafts were evident, TNF was found to be significantly lower solely in the MP compared the glycine group ($P = 0.01$).

Graft recipient survival

Because survival of the transplant recipient is the primary goal of any organ pretreatment strategy, we examined survival after KC-inhibitor use. Although direct graft function indicators, such as bile flow did not differ between the groups at 90 min after graft reperfusion, log-rank statistics on Kaplan Meier survival plots (Fig. 6) revealed a critical advantage of survival in animals receiving grafts pretreated with glycine, as compared with animals receiving grafts pretreated with $GdCl_3$ ($P = 0.045$), MP ($P = 0.014$) or saline ($P = 0.036$). Therefore, our direct comparison of the various KC-inhibitors indicates that glycine is most effective at preserving critical liver graft functions determining long-term survival.

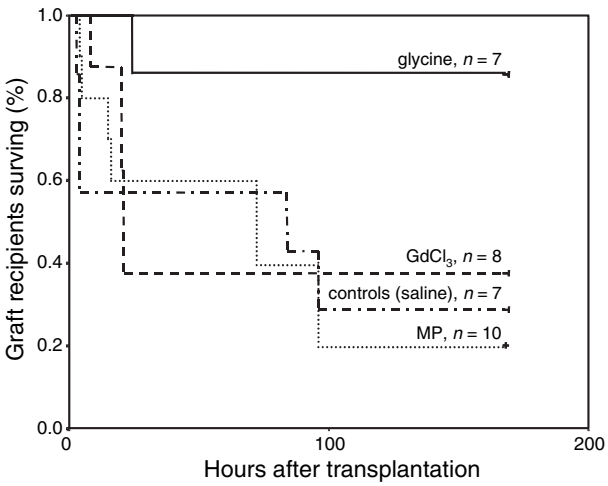

Figure 6 Glycine pretreatment was accompanied by significantly improved recipient survival, as compared with all the other groups. Kaplan–Meier estimations and log-rank comparisons, significance level 0.05.

Table 1. General data.

	$GdCl_3$ ($n = 9$)	MP ($n = 9$)	Glycine ($n = 7$)	Controls ($n = 7$)
IVM experiments				
Donor weight (g)	179.4 ± 9.6	186.6 ± 5.5	195.5 ± 7.8	196.8 ± 7.7
Recipient weight (g)	213.8 ± 16.5	215.6 ± 10.1	205.5 ± 5.5	212.6 ± 4.2
Graft weight (g)	6.9 ± 0.4	7.7 ± 0.3	7.8 ± 0.3	8.4 ± 0.2
Anhepatic time (min)	14.8 ± 0.4	14.7 ± 0.6	13.3 ± 0.4	14.9 ± 0.6
Arterialization delay (min)	5.5 ± 0.8	3.4 ± 0.5	3.5 ± 0.3	4.4 ± 0.6
Mean arterial pressure (mmHg)	64.6 ± 2.1	61.5 ± 2.6	58.4 ± 5.4	64.9 ± 3.3
Survival experiments	($n = 10$)	($n = 9$)	($n = 10$)	($n = 8$)
Donor weight (g)	175.0 ± 9.8	188.2 ± 6.3	188.8 ± 3.7	192.4 ± 13.0
Recipient weight (g)	201.2 ± 9.4	207.8 ± 6.9	214.7 ± 7.3	197.8 ± 8.6
Graft weight (g)	7.0 ± 0.4	9.2 ± 0.3	7.8 ± 0.2	8.0 ± 0.6
Anhepatic time (min)	13.7 ± 0.4	14.8 ± 0.7	13.9 ± 0.5	12.7 ± 0.9
Arterialization delay (min)	6.1 ± 1.5	3.7 ± 0.6	6.1 ± 1.0	3.1 ± 0.5

Data presented as mean ± SEM. No significant differences between all groups. n = numbers of individuals in each group.

Discussion

Recent evidence suggests that inactivation or destruction of KCs could serve as an effective regimen to overcome hepatic organ dysfunction or failure [17,19,22,27]. The present study is the first to directly compare the effects of $GdCl_3$, MP, or glycine pretreatment on post-transplant liver functions. We demonstrate that microvascular graft injury is reduced by KC inactivation early after liver transplantation following 24 h of graft storage. However, survival after liver transplantation is solely improved significantly in glycine pretreated recipients, which might be due to different effects of $GdCl_3$, MP, or glycine on apoptosis of sinusoidal lining cells and hepatocytes, as well as on the potential of graft regeneration after severe ischemia and reperfusion induced injury. These results strongly support the use of glycine as a protective drugs in liver transplantation. Furthermore, it sustains studies on warm ischemia and reperfusion and acetaminophen-induced liver injury [20,21,28] which query previous observations of beneficial effects of KC destroying agents.

Our side-by-side comparison of these three KC inhibitors revealed important differences in their effects on KCs. One difference is that the number of KC was only reduced after $GdCl_3$ and MP treatment, although phagocytic activity for fluorescent latex particles was found to be reduced in all three $GdCl_3$, MP, or glycine pretreated groups.

Therefore, our results suggest that glycine downregulates KC activity, but does not alter the number of KCs in liver grafts. Interestingly, glycine pretreatment of grafts was the only KC inhibitor strategy that significantly prolonged recipient survival.

Our finding of improved animal survival only with glycine is in contrast with previous investigations showing similar effects of $GdCl_3$ and glycine [22]. It is notable, however, that in the other investigations, liver grafts received gentle manipulation and were only held in storage short-term (1 h), or were examined under isolated perfusion conditions. Therefore, previous experiments did not apply as much stress to the explanted liver before and after transplantation. It is likely, therefore, that longer-term storage conditions in our experiments increase the sensitivity of the model system to possible differences between KC inhibitor treatment regimens. In fact, macrohemodynamic conditions, recipient age, body weight, liver graft weight, and time for graft revascularization showed no statistical difference between the groups which ensures comparability of the results. Also, to avoid practical variations, the KC inhibitors used were prepared and applied as previously described [15,22].

Analysis of liver microcirculation in our study revealed some subtle differences between the different KC inhibitors. Indeed, it has already been shown that KC inactivation with $GdCl_3$ significantly reduces microvascular perfusion failure in hepatic sinusoids using the same rat model [27], and homogenization of microvascular perfusion in gently manipulated livers has been demonstrated with $GdCl_3$ or glycine [22]. Consistent with these studies, we observed a reduced number of nonperfused acini after pretreatment with $GdCl_3$ and MP, but this effect was most impressive with glycine. However, this effect was not reproducible with any of the 3 KC inhibitors when we analyzed sinusoidal graft perfusion in our experiments. This observation may be due to the fact, that distribution of fluorescent components sinusoidal perfusion allows the quantification exclusively in perfused acini, when intravital microscopy is applied. As a consequence sinusoidal perfusion measurements in intravital microscopy experi-

ments can only be interpreted in the context of acinar perfusion failure. Nonetheless, hepatic acini containing multiple single hepatic sinusoids (in our experiments 3–12, mean: 7 sinusoids) represent a much larger microvascular unit than do sinusoids. Microvascular perfusion failure of complete acini may affect a much larger volume of hepatic tissue than do individual sinusoids. Indeed, our observation that glycine pretreated animals showed the best survival after 7 days supports this conclusion.

Leukocyte adherence and accumulation in liver grafts early after reperfusion has been postulated as a likely contributor to ischemia reperfusion injury by being a source of potentially toxic mediators [4,7,24]. The significance of leukocyte adherence has been questioned although [29], and intravital microscopy studies of transplanted livers after prolonged cold ischemia failed to document beneficial effects of blocking leukocyte adherence on early graft function [30]. In addition, liver regeneration after 70% partial hepatectomy has been shown to be dependent on an intact recruitment sequence of leukocyte adherence [31]. In this model, KCs may play a fundamental role in the recruitment of leukocytes via release of cytokines, in particular TNF-α and IL-6 [31]. Furthermore, this mechanism appears to be mandatory for regeneration of reduced size rat liver grafts as well as severely injured livers after CCl_4 exposition [32,33]. However, the effect of KC depletion or modulation on cell survival and capacity of regeneration on liver grafts severely injured by cold ischemia is yet unknown. This led us to examine in particular leukocyte infiltration and TNF-α expression in liver grafts of the present experiments. As assessment of leukocyte adherence by means of intravital microscopy may provide information only on intravascular flow and adherence behavior of leukocytes, myeloperoxidase activity in hepatic tissue was quantified by real time polymerase chain reaction (quantitative RT-PCR). In paralleled examinations, TNF-α expression was analyzed. Interestingly, in complete accordance with the mentioned studies, [32,33] MPO as well as TNF-α expression was higher after glycine treated liver grafts as after depletion of KCs with $GdCl_3$ or MP, probably indicating the preserved ability for regeneration via TNF-α dependent leukocyte recruitment following glycine pretreatment. Previous observations with examining the potential of hepatic regeneration after elimination of KCs with $GdCl_3$ [31] and MP [34] clearly demonstrate the detrimental effect of KC depletion, and our data on TNF-α levels und leukocyte infiltration together with the results of animal survival measured 7 days after transplantation would favor this assumption.

Nonetheless, differences of cellular graft function or intrahepatic cell death after transplantation could be expected, if the difference in graft survival was determined by variations in the potential of liver regeneration after transplantation in dependence on KC depletion or modulation. We monitored early liver graft function by hepatocellular excretion, which, in contrast to serum levels of hepatocellular enzymes or hepatic mediators, indicates graft function independent of perfusion irregularities [8]. Bile flow over 90 min, was not affected by any of the three treatment regimens. A slightly, but not significant, improved bile salt excretion after glycine pretreatment might indicate some degree of hepatocellular protection in the glycine-, as compared with the MP- or $GdCl_3$-group. However, the striking differences in survival between the groups may not be explained by effects on graft excretory function.

Interestingly, although all three KC-inhibiting agents reduced apoptotic cell death as early as 90 min after graft reperfusion, $GdCl_3$ and MP treatment only delayed the development of cell death. Analysis of TUNEL-sections of liver grafts retrieved at 6 and 9 h following reperfusion further suggest, that KC inactivation plus direct cellular protection by glycine might result in a profounder protection of liver grafts, than depletion of KCs by $GdCl_3$ and MP. This finding is substantiated by our results on caspase activation showing a strong signal for cleaved (activated) caspase 3 after $GdCl_3$ and MP treatment, but not after glycine treatment. In untreated controls however, induction of cell death leading to graft loss of 70% of recipients, may either be strongly accelerated or, more likely, independent of caspase 3 cleavage. This latter mechanism would be supported by the necrapoptosis hypothesis [35], presuming mixed modes of cell death (apoptosis and necrosis) after cold ischemia and reperfusion of liver grafts. Cell death via necrosis occurs rapidly after graft reperfusion [6], whereas apoptosis takes several hours to develop [5]. This could explain the high numbers of TUNEL positive cells in the control group without elevation of cleaved caspase 3. On the other hand, apoptosis is a dynamic process, and caspase 3 cleavage was found strongly augmented early after reperfusion of liver grafts following 24 h of cold storage as compared with solely cold stored livers [36]. Thus, detection of TUNEL positive cells at 90, 360 and 540 min after liver graft reperfusion may simply represent morphologic expression of preceding molecular mechanisms. The elevation of TNF-α in control and glycine pretreated liver grafts compared with KC-depleted grafts not necessarily contradicts the findings of apoptotic cell death. Overwhelming TNF-α levels indeed strongly induce apoptosis and necrosis [35,37]. However, TNF-α stimulation may exert multiple distinct effects, including the induction of NF-κB, which is related to a cellular regenerative response after several types of injury [33,38]. Thus, again, glycine pretreatment may preserve the potential for

TNF-α dependent regeneration processes superior to $GdCl_3$ or MP. Together, the present data emphasize the previously assumed beneficial effects of glycine-preconditioning of liver grafts [17,19] and as the first outline substantial advantages in contrast to KC depleting agents. The glycine-effects are most likely linked to prevention microvascular injury and preserved cellular regeneration potential and reduced apoptotic and necrotic cell death, resulting in increased liver graft survival.

In summary, glycine is a highly effective substance for preventing ischemia and reperfusion-related liver graft injury. As protective effects on kidney, heart, intestine beside of the hepatoprotective [17], and no adverse effects with its use are reported, glycine may therefore have multiple advantages for clinical use and should be broadly considered to reduce the incidence of early liver graft dysfunction.

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References

1. Ploeg RJ, D'Alessandro AM, Knechtle SJ, *et al.* Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. *Transplantation* 1993; **55**: 807.
2. Pirenne J, Gunson B, Khaleef H, *et al.* Influence of ischemia-reperfusion injury on rejection after liver transplantation. *Transplant Proc* 1997; **29**: 366.
3. Land W, Schneeberger H, Schleibner S, *et al.* The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants. *Transplantation* 1994; **57**: 211.
4. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992; **53**: 957.
5. Natori S, Selzner M, Valentino KL, *et al.* Apoptosis of sinusoidal endothelial cells occurs during liver preservation injury by a caspase-depedent mechanism. *Transplantation* 1999; **68**: 89.
6. Caldwell Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ. Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. *Hepatology* 1991; **13**: 83.
7. Jaeschke H. Preservation injury: mechanisms, prevention and consequences. *J Hepatol* 1996; **25**: 774.
8. Sankary HN, Foster P, Brown E, Hart M, Williams JW. A comparison of Collins and UW solutions for cold ischemic preservation of the rat liver. *J Surg Res* 1991; **51**: 87.
9. Sumimoto K, Inagaki K, Yamada K, Kawasaki T, Dohi K. Reliable indices for the determination of viability of grafted liver immediately after orthotopic transplantation. Bile flow rate and cellular adenosine triphosphate level. *Transplantation* 1988; **46**: 506.
10. Borghi-Scoazec G, Scoazec J, Durand F, *et al.* Apoptosis after ischemia-reperfusion in human liver allografts. *Liver Transpl Surg* 1997; **3**: 407.
11. Weiss GB, Goodman FR. Effects of Lanthanum on contraction, calcium distribution and Ca[45] movement in intestinal smooth muscle. *J Pharmacol Exp Ther* 1969; **160**: 46.
12. Hardonk MJ, Dijkhuis FWJ, Hulstaert CE, Koudstaal J. Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukoc Biol* 1992; **52**: 296.
13. Di Luzio NR, Wooles WR. Depression of phagocytic activity and immune response by methyl palmitate. *Am J Physiol* 1964; **206**: 939.
14. Al Tuwaijri A, Akdamar K, Di Luzio NR. Modification of galactosamine-induced liver injury in rats by reticuloendothelial system stimulation or depression. *Hepatology* 1981; **1**: 107.
15. Marzi I, Cowper K, Takei Y, Lindert K, Lemasters JJ, Thurman RG. Methyl palmitate prevents Kupffer cell activation and improves survival after orthotopic liver transplantation in the rat. *Transpl Int* 1991; **4**: 215.
16. Ikejima K, Qu W, Stachlewitz RF, Thurman RG. Kupffer cells contain a glycine gated chloride channel. *Am J Physiol* 1997; **272**: G1581.
17. Zhong Z, Wheeler MD, Li X, *et al.* L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. *Curr Opin Clin Nutr Metab Care* 2003; **6**: 229.
18. den Butter G, Lindell SL, Sumimoto R, Schilling MK, Southard JH, Belzer FO. Effect of glycine in dog and rat liver transplantation. *Transplantation* 1993; **56**: 817.
19. Schemmer P, Bradford BJ, Rose ML, *et al.* Intravenous glycine improves survival in rat liver transplantation. *Am J Physiol* 1999; **276**: G924.
20. Imamura H, Sutto F, Brault A, Huet PM. Role of Kupffer cells in cold ischemia and reperfusion injury of rat liver. *Gastroenterology* 1995; **109**: 189.
21. Kobayashi T, Hirano KI, Yamamoto T, *et al.* The protective role of Kupffer cells in the ischemia-reperfused rat liver. *Acta Histol cytol* 2002; **65**: 251.
22. Schemmer P, Connor HD, Arteel GE, *et al.* Reperfusion injury in livers due to gentle in situ manipulation during harvest involves hypoxia and free radicals. *J Pharmacol Exp Ther* 1999; **290**: 235.
23. Kamada N, Calne RY. Orthotopic liver transplantation in the rat. Technique using cuff for portal vein anastomosis and biliary drainage. *Transplantation* 1979; **28**: 47.
24. Post S, Palma P, Rentsch M, Gonzalez AP, Menger MD. Differential impact of Carolina rinse and University of Wisconsin solutions on microcirculation, leukocyte adhesion, Kupffer cell activity and biliary excretion after liver transplantation. *Hepatology* 1993; **18**: 1490.

25. Turley SD, Dietschy JM. Re-evaluation of the 3 alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J Lipid Res* 1978; **19**: 924.
26. Dan C, Wake K. Modes of endocytosis of latex particles in sinusoidal endothelial and Kupffer cells of normal and perfused rat liver. *Exp Cell Res* 1985; **158**: 75.
27. Schauer RJ, Bilzer M, Kalmuk S, *et al.* Microcirculatory failure after rat liver transplantation is related to Kupffer cell-derived oxidant stress but not involved in early graft dysfunction. *Transplantation* 2001; **72**: 1692.
28. Ju C, Reilly TP, Bourdi M, *et al.* Protective role of Kupffer cellsin acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol* 2002; **15**: 1504.
29. Otto G, Hofheinz H, Hofmann WJ, Manner M. Questionable role of leukocyte sticking in the pathogenesis of preservation damage. *Transplant Proc* 1991; **23**: 2385.
30. Rentsch M, Post S, Palma P, Lang G, Menger MD, Messmer K. Anti-ICAM-1 blockade reduces WBC adherence following ischemia and reperfusion but does not improve early grafts functioning rat liver transplantation. *J Hepatol* 2000; **32**: 821.
31. Selzner N, Selzner M, Odermatt B, Tian Y, van Rooijen N, Clavien PA. ICAM-1 triggers liver regeneration through leukocyte recruitment and Kupffer cell-dependent release of TNF-alpha/IL-6 in mice. *Gastroenterology* 2003; **124**: 692.
32. Selzner N, Selzner M, Tian Y, Kadry Z, Clavien PA. Cold ischemia decreases liver regeneration after partial liver transplantation in the rat: A TNF-alpha/IL-6-dependent mechanism. *Hepatology* 2002; **36**(4 Pt 1): 812.
33. Yamada Y, Fausto N. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. *Am J Pathol* 1998; **152**: 1577.
34. Kato K, Kazuhiko O, Kato J, Kasai S, Mito M. The immuno-stimulant OK-432 enhances liver regeneration after 70% hepatectomy. *J Hepatol* 1995; **23**: 87.
35. Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology* 2003; **125**: 1246.
36. Rentsch M, Beham A, Iesalnieks I, Mirwald T, Anthuber M, Jauch KW. Impact of prolonged cold ischemia and reperfusion on apoptosis, activation of caspase 3, and expression of bax after liver transplantation in the rat. *Transplant Proc* 2001; **33**: 850.
37. Brock RW, Lawlor DK, Harris KA, Potter RF. Initiation of remote hepatic injury in the rat: interactions between Kupffer cells, tumor necrosis factor-alpha, and microvascular perfusion. *Hepatology* 1999; **30**: 137.
38. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappa B activation prevents cell death. *Cell* 1996; **87**: 565.